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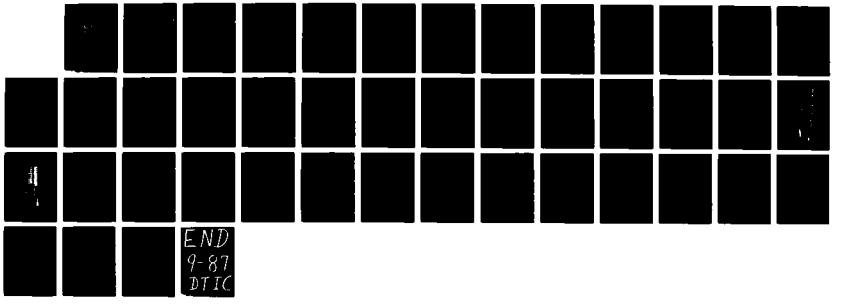

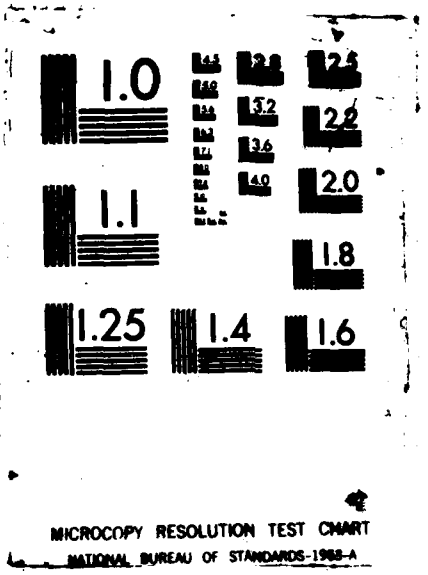
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The RNA modification enzyme, tRNA pseudouridine synthase I (PSUI) has been isolated in 95% purity from an Escherichia coli strain harboring a multicopy plasmid with a 2.3kb insert for the hisT operon. Its molecular size, amino acid composition and N-terminal sequence correspond to those predicted by the structure and expression of the hisT gene. Enzyme activity, as measured by a ^{3}H release assay, is unaffected by pretreatment if PSUI with micrococcal nuclease and is optimized by the addition of a monovalent cation and thiol reductant. The activity is inhibited by all tRNA species tested, including substrates, modified tRNAs, non substrates and tRNAs containing 5-fluorourdine. Binding of PSUI occurs with both susbtrates and non substrates tRNAs and does not require a monovalent cation. Our finding are consistent with a multi-step mechanism whereby PSUI first binds non-specifically, then forms transient covalent adducts with tRNA substrates. Keywords: charts; graphs; tables (data)

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PURIFICATION, STRUCTURE AND PROPERTIES OF ESCHERICHIA COLI tRNA PSEUDOURIDINE SYNTHASE I*

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RUNNING TITLE

Properties of E. coli tRNA Pseudouridine Synthase I

SUMMARY

The RNA modification enzyme, tRNA pseudouridine synthase I (PSUI) has been isolated in 95% purity from an Escherichia coli strain harboring a multicopy plasmid with a 2.3 kb insert from the hisT operon. Its molecular size, amino acid composition and N-terminal sequence correspond to those predicted by the structure and expression of the hisT gene. Enzyme activity, as measured by a ^{3}H release assay, is unaffected by pretreatment of PSUI with micrococcal nuclease and is optimized by the addition of a monovalent cation and thiol reductant. The activity is inhibited by all tRNA species tested, including substrates, modified tRNAs, non-substrates or tRNAs containing 5-fluorouridine. Binding of PSUI occurs with both substrate and non-substrate tRNAs and does not require a monovalent cation. Our findings are consistent with a multi-step mechanism whereby PSUI first binds non-specifically, then forms transient covalent adducts with tRNA substrates.

In the absence of other proteins, purified PSUI forms ψ at all three modification sites known to be affected in hisT mutants. The 36.4 kDa polypeptide product of the gene adjacent to hisT, whose translation is linked to that of PSUI, is not a functional subunit for PSUI activity, or a separate synthase acting at one of the three loci.

Pseudouridine (5-ribosyl uracil)(ψ) is one of the few -C-C- nucleosides found in natural sources, and is present in many classes of ribonucleic acid. This modified nucleoside is abundant in cytoplasmic and organelle transfer RNAs (1); it is also present in the large bacterial and eukaryotic ribosomal RNAs (2-6), the 5S RNA from yeasts(7), the 5.8S RNA from most eukaryotic ribosomes (8,9), all of the small nuclear (sn) RNAs (U_1-U_6) and other eukaryotic nuclear RNA species(10). It is clear that this diverse group of modifications is produced by a family of RNA ψ synthases of highly selective site specificity. As the most extensively studied member of this family, PSUI offers the best opportunity for analyzing the genetics, enzyme mechanism and molecular interactions of this class of modification enzymes.

PSUI was originally identified as the product of the hisT gene of Salmonella typhimurium (11-13) and E. coli (14,15). hisT strains accumulate a group of undermodified, but otherise mature tRNA isoacceptors (16) which can serve as modifiable substrates to identify and measure the catalytic activity of PSUI (13). PSUI activity has been detected in extracts from coliform bacteria (13), yeasts, cultured mammalian cells, tumor cell lines and both adult and embryonic tissues (17). Although partial purification of PSUI from several sources has been reported (18-20), this protein is present in small quantities in most cells, and has not been isolated in sufficiently pure form for structural studies.

In order to amplify the production of PSUI by recombinant DNA methods, the E. coli hisT gene was first identified in the Carbon-Clarke plasmid pLC28-44 and a 2.3 kb insert containing the gene was subcloned into pBR322 (21). Cells transformed with this plasmid (ψ_{300}) were found to overexpress PSUI activity by a factor of 15-20 (21). The localization of the hisT gene in a multi-component operon and the organization and sequence of the 2.3 kb insert have been reported (21,22).

In this report, we describe the isolation of virtually homogeneous PSUI from strains of E. coli transformed with plasmid ψ_{300}, in which the production of PSUI is amplified 20-fold. The results validate the primary structure of PSUI, as deduced from the DNA sequence, and describe its general enzymatic features. Although the genes for PSUI and an upstream 36.4 kDa protein are transcriptionally linked and translationally coupled, the proteins appear to be functionally independent.

METHODS AND MATERIALS[1]

EXPERIMENTAL RESULTS

Purification of PSUI: Most of the results presented in this paper were obtained with PSUI prepared from E. coli 294 bearing plasmid ψ_{300}: in these cells, ca. 95% of the enzyme was derived from expression of the plasmid hisT gene, rather than the hisT gene of the host chromosome. The procedure for isolation of PSUI, as outlined in Table I, was adapted from the method of Arena et al. (20) for enrichment of the enzyme from S. typhimurium. A useful property of PSUI is its precipitability in the streptomycin sulfate step, which provides an effective purification with high recovery. This behavior probably arises from the high affinity and co-precipitation of PSUI with various RNAs. The final recovery of activity of PSUI was 10%, with an overall enrichment of 700-fold[2]. The protein was quite stable in the cold; in storage buffer containing 50% glycerol (w/v), 50% of the activity survived after two years at 2^{o} and at -20^{o}. The PSUI preparations contained traces of an endonuclease activity acting on single-stranded cytidine residues in tRNA. This activity has not been characterized further.

Physical Properties and Correlation with the hisT Gene Sequence: The concentrated Sephadex G-100 fraction showed one major band, accounting for

95% of the protein, after electrophoresis in 15% polyacrylamide-SDS gels (Figure 1C). The mobility of the PSUI subunit was virtually identical with that of carbonic anhydrase (molecular mass 31,000 Da). This subunit size agreed with that of the hisT product formed in maxicells, and with an in vitro transcription-translation product of plasmid ψ_{300} DNA (21). It also corresponded to the molecular mass for PSUI (30,399 daltons, 270 amino acids) predicted by the hisT gene sequence (22) .

The amino-terminal sequence of PSUI was determined with a gas-phase sequenator[3]. The results showed that the sequence of residues 1-10 of the PSUI subunit, H_2NSer-asp-gln-gln-gln-pro-pro-val-tyr-lys-, is identical to that of amino acids 2-11 deduced from the hisT gene sequence (22). This result unambiguously validates the correspondence between the hisT gene sequence and the amino acid sequence of PSUI and indicates that the amino-terminal methionine residue is processed after synthesis of the enzyme. The deduced amino acid sequence is shown in Figure 2, and illustrates the location of acidic, basic and cysteine residues in the protein.

The amino acid composition of PSUI was consistent with that deduced from the coding sequence of the hisT gene (data not shown) and validates the assigned open reading frame for the hisT gene product (22).

Kinetic Properties of PSUI: The kinetic features of the PSUI reaction have been analyzed by [^{3}H] release from bulk [5-^{3}H-pyrimidyl] hisT tRNA as substrate (18). The basic reaction requirements are shown in Table II and indicate that maximal rates of ^{3}H release take place in the presence of a monovalent cation (optimum range for NH_4^+ 60-120 mM) and a thiol reductant. No significant effect was produced by the presence of various nucleotide additives, free ψdo and ψp. The reaction was specific for [5-^{3}H-pyrimidyl] labeled hisT tRNA, which contains modifiable uridine residues, but did not

take place with wild-type tRNA, in which the modifications were already synthesized.

The thiol requirement can be met by common sulfhydryl reductants such as cysteine, β-mercaptoethanol, dithiothreitol; in vivo, glutathione is the likely reductant for the enzyme[4]. The involvement of a thiol was further emphasized by the effects of thiol-specific reagents, which progressively inhibited the reaction with increasing concentrations (Table III). Most cogently, pre-incubation of PSUI with PCMB, iodoacetate, or DTNB irreversibly inactivated the enzyme. Since pre-treated enzyme was subsequently assayed with excess thiol, these findings point to a significant role for cysteine residue(s) in catalysis or maintenance of the active structure of PSUI.

Effects of Nucleosides, Nucleotides and Nucleic Acids: In order to test whether an RNA component might be involved in the catalysis, a sample of PSUI was first treated with micrococcal nuclease, then assayed for ^{3}H release after completely complexing the Ca++ with EGTA. PSUI was fully active after micrococcal nuclease treatment, indicating that the native enzyme does not contain a catalytically significant RNA.

The effects of other nucleic acid constituents on PSUI activity are shown in Tables IV and V. At equimolar concentrations or higher, all tested tRNAs inhibited the ^{3}H release from labeled bulk hisT tRNA. This occurred with tRNA substrates (hisT $tRNA^{Phe}$, hisT $tRNA^{His}$), with products of the enzyme reaction (wild-type $tRNA^{Phe}$, wild-type $tRNA^{His}$, yeast $tRNA^{Phe}$) and even with tRNAs that are neither substrates nor reaction products ($tRNA^{Glu}$, $tRNA^{Met}f$ and $tRNA^{Asp}$). Each of these RNA species lowered the enzyme activity but with significant quantitative differences. Thus, the action of PSUI involves both specific and non-specific interactions with tRNA.

Bulk FU-tRNA, whether from a wild-type or hisT source was a powerful

inhibitor of the reaction (Table V). At equimolar concentrations of FU-tRNA and labeled tRNA substrate, PSUI activity was reduced by 85-90%. This effect was virtually irreversible, since pretreatment of the enzyme with FU-tRNA was equally inhibitory. Table V also shows that the losses of enzyme activity which occasionally attend preincubation of PSUI, were totally prevented by the presence of a single species of tRNA or a bulk tRNA preparation. The inclusion of a thiol during preincubation was ineffective.

Purified PSUI was also tested for the ability to catalyze the following: (1) exchange of exogenous uracil with wild-type or hisT tRNA; (2) ^{3}H release from [5-^{3}H]labeled uracil, uridine, or UTP; (3) presence of tRNA-independent pseudouridylate synthase activity (24)(also assayed under optimum conditions for PSUI). None of these reactions was detected.

Binding of PSUI to RNA (Figures 3,4): For further definition of the interactions of PSUI with tRNA, we have employed an electrophoretic procedure to demonstrate the formation of macromolecular complexes. When a fixed quantity of labeled tRNA substrate (hisT $tRNA^{Phe}2$) was incubated with increasing concentrations of PSUI (Fig. 3A), a group of four molecular complexes was produced of progressively lower mobility than that of the tRNA substrate. Coomassie Blue staining revealed the presence of PSUI in the complexes in addition to labeled tRNA. A similar group of complexes was produced by titration of labeled hisT $tRNA^{His}$ (Fig. 3A, lanes 10-12). The complexes formed from hisT $tRNA^{Phe}2$ were extracted from the gel with buffered phenol and the RNA in each was reisolated and analyzed electrophoretically. Each complex contained RNA of the same mobility as that of the original tRNA substrate (data not shown). Thus, the multiplicity of complexes was not due to differential binding of RNA fragments to PSUI, but reflected distinct molecular associations of tRNA and enzyme. Since most of the tRNA was

recovered by phenol extraction of the complexes, the RNA must have been non-covalently bound to the enzyme, or covalently bound in a readily reversible form.

Complex formation was not limited to modifiable tRNA substrates but also occurred with pseudouridylated tRNAs (wild-type $tRNA^{His}$, wild-type $tRNA^{Phe}_2$ and yeast $tRNA^{Phe}$), and with tRNAs that were not substrates or reaction products of PSUI (S. typhimurium $tRNA^{Asp}$)(Figure 3A). PSUI-tRNA complexes were demonstrable within 15 seconds of incubation at 15^o or 25^o, but were not formed at 0^o, or when BSA was substituted for PSUI (Fig. 4A). Further analysis showed that PSUI-tRNA complexes were formed in the absence of NH_4^+ (Figure 3B), in contrast to its absolute requirement for ^3H release (Table III). Omission of thiol, or incubation or pre-incubation with thiol inhibitors drastically reduced complex formation (Figure 3B).

Once a tRNA was bound to PSUI, it was firmly but reversibly retained. The complexes were stable in buffer at 25^o for at least 30 min with little or no dissociation of the tRNA. A significant percentage of labeled tRNA initially complexed with PSUI remained bound, even after a subsequent chase with a 25-fold excess of another tRNA (Figure 4). By employing labeled displacing tRNA, it was found to be distributed among the complexes in the same manner as the tRNA originally bound. As a first approximation, the ability of a tRNA to displace a pre-bound species paralleled its ability to inhibit the ^3H release reaction. E.g., when hisT $tRNA^{Phe}_2$ was initially bound to PSUI, it was more readily displaced by wild-type $tRNA^{Phe}_2$ than by $tRNA^{Asp}$ (a weak inhibitor of the reaction)(Fig. 4C). Similarly, when $tRNA^{Asp}$ was first bound to PSUI, it was readily displaced by both wild-type and hisT $tRNA^{Phe}_2$. While these relationships require further refinement, it is evident that PSUI essentially complexes with all tRNAs and must recognize structural

features that are common to these molecules. But, since individual tRNAs differ in the capacity to bind to PSUI or to displace pre-bound RNA, some property of modifiable tRNA substrates must account for their selectivity.

Modification of hisT tRNA Isoacceptors by Purified PSUI: Genetic and molecular analysis of the hisT gene have shown that it is closely linked to an adjacent upstream gene ("usg") which specifies a 36.4 kd polypeptide of unknown function (21,22). The hisT operon contains several additional genes (22, 30) and appears to be subject to complex regulatory control. The close linkage of the hisT and "usg" genes and their translationally coupled expression (22) raises the issue of whether their protein products are functionally related.

Although PSUI showed catalytic activity (^3H release) in the absence of the 36.4 kd component, which is removed during purification, it was important to assess whether ψ was actually formed by the action of the enzyme on tRNA substrates. (I.e., ^3H release might be a partial reaction in the overall modification sequence). The data in Figure 5 illustrate the results of such a test. Bulk unfractionated tRNA from hisT strain, E. coli FB 105, was treated with PSUI so as to modify completely all the accessible uridine sites. Under these conditions, the maximal level of ^3H release from the hisT tRNA substrate was proportional to the tRNA concentration, as expected (data not shown). PSUI-modified tRNA preparations or untreated controls were aminoacylated with [^3H]tyrosine, -histidine or -leucine and were analyzed on an RPC-5 column, by co-chromatography with wild-type tRNA markers aminoacylated with the corresponding [^{14}C]amino acids. The results (Figure 5) show conclusively that purified PSUI modifies all of the hisT isoacceptors of $tRNA^{His}$, $tRNA^{Tyr}$ and $tRNA^{Leu}$ to products which are chromatographically indistinguishable from the respective wild-type species. These three groups

of isoacceptors contain all the known topological sites for ψ modification of residues 38, 39 and 40. Since purified PSUI completely modifies these hisT isoacceptors, the activity of PSUI is functionally independent of the products of the "usg" gene and the other genes of the operon. This result is in complete accord with gentic experiments of Arps et al. (22), which demonstrate the structural and functional independence of PSUI and the other products of the hisT operon.

DISCUSSION

Physical Characterization of PSUI and Correlation of its Structure with that of the hisT Gene. The genetic organization and DNA sequence of a cloned 2.3 kb fragment containing the E. coli hisT gene have recently been determined (21,22). The properties of PSUI reported here provide direct structural evidence correlating the biochemical and genetic features of the enzyme. The molecular size of the PSUI subunit (31,000 Da) agrees closely with that predicted from the gene sequence (30,399 Da) and corresponds to that of the hisT gene product expressed in vivo and in vitro (21). The N-terminal amino acid sequence of PSUI also substantiates the presumed translational initiation site and independently confirms the identity, N-terminal sequence and localization of the hisT gene. The amino acid composition of PSUI is also consistent with that expected from translation of the open reading frame for the complete length of the hisT gene (22).

Arena et al. have described the properties of PSUI, purified 1000-fold from S. typhimurium (20). Its kinetic features resemble those of E. coli PSUI, but a molecular mass of 50,000 daltons was estimated for the Salmonella enzyme subunit. The reasons for the difference in these molecular size estimates are not obvious. In their study, it was also reported that exposure of PSUI to tRNA led to dimerization of the enzyme, without tRNA binding in

the complex. In contrast, we find that PSUI forms a group of molecular complexes whose structures vary with the relative concentrations of enzyme and RNA, but that tRNA is present in each. Our results have not determined whether the PSUI-tRNA complexes also include covalent adducts. These issues and the exact mechanism of complex formation should be clarified by further analysis of their formation and dissociation.

The hisT gene is part of a complex, differentially expressed operon which contains at least four genes (21,22,30). Until recently, the hisT gene was the only one with a known function, but Winkler and Arps (30) have now identified the gene upstream from "usg" as one which codes for an enzyme in pyridoxine biosynthesis (30). Our results clearly show that the hisT gene product, PSUI, is functionally independent of "usg" and the other products of the operon, and agree with the conclusions derived from genetic studies (22).

The content and distribution of basic amino acids in PSUI are striking (Figure 2). The enzyme contains 11 lysine, 20 arginine and 11 histidine residues in a total of 270 amino acids. Many of these are clustered in two large domains which are almost devoid of acidic amino acids, extending between residues 108-134 and residues 171-206. These regions could provide strongly cationic patches for the attachment of PSUI to specific regions of the tRNA backbone. Hydrophobicity plots based on the protein sequence (31) showed no evidence of strongly hydrophobic domains, in keeping with the localization of PSUI in the cytosol.

Comparison of the hisT gene sequence with the Genbank data base revealed no significant homologies, even with the genes for other enzymes which are reactive at C_5 of uracil (thymidylate synthase and aminoacyl synthetases). The deduced amino acid sequence of PSUI was also compared with the Dayhoff protein sequence bank, with similar results. A search for internal

duplications within the hisT gene and PSUI protein sequences revealed a possible short region of DNA and amino acid sequence homology encompassing two of the three cysteine residues, (Cys154 and Cys169) which contain the common sequence, -Arg-Ala-X-Gln-Cys- . While this homology is limited, it will be interesting to learn whether it has a functionally significant role.

There appears to be a significant conservation of sequence in the structure of PSUI among various prokaryotic organisms, based on hybridization of genomic libraries with hisT probes[5]. Although common structural features might also be anticipated for PSUI and other tRNA ψ synthases, preliminary experiments have failed to reveal the presence of other hybridizable genes in coliform organisms[5].

Site Specificity and Mechanistic Considerations: The tRNAs of coliform bacteria contain ψ at seven positions of the molecule (Figure 6). In a survey of S. typhimurium tRNA isoacceptors, we have found that all tRNA modifications are present in hisT mutants, except for those at residues 38, 39 and 40[6]. The action of PSUI is directed solely to the modification of uridine residues at these three loci in specific tRNA isoacceptors. Virtually no coliform tRNA contains an unmodified uridine at these locations (1). It follows that PSUI is not involved in the synthesis of ψ at positions 13, 32, 55 and 65 of coliform tRNA, nor does it participate in the formation of ψ in the ribosomal RNA of these organisms. These findings also rule out the prospect that PSUI might function as as a catalytic subunit for any of the other tRNA pseudouridylation enzymes in these organisms.

PSUI modifies susceptible uridine residues in the anticodon region without an RNA or other protein recognition component. While PSUI can modify otherwise mature hisT isoacceptors, it can also pseudouridylate tRNA precursor species (32), including in vitro transcripts which lack other

modifications (33).

Mechanistically, the reaction must involve at least four steps: (1) binding of PSUI to the tRNA; (2) cleavage of the base-sugar glycosidic bond; (3) a 180^{o} rotation of the base relative to the ribofuranosyl ring; and (4) reformation of the -C-C- bond of ψ . Since PSUI binds to any tRNA under conditions which do not lead to ^{3}H release, it is likely that this non-specific step initiates the reaction and brings the catalytic site into proximity with the anticodon region of the tRNA. Experiments are in progress to localize the sites of tRNA interaction with PSUI.

The mechanism elucidated by Santi and others for thymidylate synthase (34-38) provides a conceptual model for the subsequent course of the PSUI reaction. In this view, substitutions at C_5 of the uracil ring are initiated by addition of a nucleophilic group in the protein to C_6 of the pyrimidine ring, forming a transient dihydropyrimidine-enzyme adduct. Similar adducts are found in the reaction of aminoacyl tRNA synthetases with residue U_8 of their cognate tRNA substrates (39,40). In the case of thymidylate synthase, the catalytic nucleophile is a cysteine residue (37).

Our preliminary findings described here for PSUI are compatible with such a scheme (38). The irreversible inactivation of PSUI by PCMB and iodoacetic acid points to a role for one or more of the three cysteine residues as a reactive nucleophile in the reaction. The action of FU-tRNAs as irreversible inhibitors is also consistent with this mechanism, since these could form stable or rearranged adducts. This model also accounts for the failure of exogenous uracil to equilibrate with tRNA uracil. This mechanism can be tested by reacting the enzyme with model substrates such as halogenated pyrimidine derivatives, which have been valuable in defining the mechanisms of thymidylate synthase (34-38) and aminoacyl tRNA synthetases (39,40).

B. Ames and his associates have pointed out that the 5'- regulatory region of the *his* operon mRNA can be folded to produce a structure closely resembling that of histidyl tRNA (41). They suggest that proteins which interact with tRNAs, such as PSUI, may have a regulatory role, by binding to tRNA-like structures in mRNA, thereby influencing the course of transcription or translation. The availability of highly purified PSUI and of high copy number plasmids bearing the *hisT* gene provide practical tools for tests of this hypothesis *in vivo* and *in vitro* . In this regard, the concentration of tRNA in *E. coli* is vastly in excess of that of PSUI, even in the most amplified systems for expressing PSUI. We estimate that *E. coli* contains 400-800 molecules of PSUI per genome equivalent at basal levels, as compared with 4×10^5 molecules of tRNA. Even after the 20-fold amplification obtained with plasmid ψ_{300}, the tRNA population would still be 25-50 times greater than that of PSUI. Mechanisms that envision a regulatory role of PSUI *via* a titration of tRNA-like elements would have to take into account the preponderance of competing tRNA species in the cell. However, if pseudouridylation were to occur after binding of PSUI to regulatory regions, significant effects on the mRNA structure and translation could follow. At the elevated levels of PSUI found in cells transformed with ψ_{300}, no readily discernable effects on cell growth or gene expression have been noted, although subtle changes might not have been evident.

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FOOTNOTES

[1] Portions of this paper ("Methods and Materials") are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. , cite the authors, and include a check or money order for $. per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

The abbreviations used are: BSA, bovine serum albumin; BND-cellulose, benzoylated, naphthoylated DEAE-cellulose; DHB-cellulose, N-[N'-(m-dihydroxyborylphenyl)-succinamyl]aminoethylcellulose; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); PCMB, p-chloromercuribenzoic acid; β-MCE, β-mercaptoethanol.

[2] The distribution of activity on Sephadex G-100 shows a slightly skewed profile, suggestive of possible heterogeneity in the protein. A recent procedural modification, in which gel filtration was carried out with a TSK 250 HPLC column, resolved the activity into two fractions which contained the same polypeptide subunit. The first fraction to elute contained 25% of the total activity in the two peaks.

[3] We are grateful to Dr. Russel Blacher of Applied Biosystems, Inc., for his assistance in the N-terminal sequence analysis and to Alan J. Smith of the Dept. of Biochemistry and Biophysics, Univ. of California at Davis, Davis CA, for analysis of the amino acid composition.

[4] S.J. Spengler and H.O. Kammen, unpublished observations. With S. typhimurium extracts, if the initial purification steps and enzyme

assays are carried out without added thiol, the streptomycin supernatant is found to contain a dialyzable material which lacks PSUI activity but stimulates the activity of the streptomycin pellet fraction. Cysteine, β-MCE or glutathione can replace the stimulatory material. Quantitative estimates show that the stimulatory activity of the streptomycin supernatant is completely accounted for by its glutathione content.

[5] M.E. Winkler, unpublished observations.

[6] H.O. Kammen, J.Preiss, M. Buck and E.E.Penhoet, in preparation.

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The authors acknowledge the expert technical assistance of Ms. Joan Quay. We are grateful to Dr. Barbara Vold for supplying Plaskon CTFE 2300 for preparation of RPC-5 resin; Dr. Bob Kudrna for growth and harvesting of bacterial cells; Mike Nonet for the hydropathy data for PSUI.

FIGURE LEGENDS

Figure 2. Amino acid sequence of PSUI (22) and distribution of anionic and cationic groups. The locations of arginine and lysine residues are designated by the (+) symbol; histidine residues by the (±) symbol; aspartic acid and glutamic acid residues by the (-) symbol. The three cysteine residues are indicates by asterisks. The N-terminal sequence determined on the purified protein is enclosed in the solid box and overlaps a pentapeptide sequence reported earlier (22).

Figure 3. Formation of complexes between PSUI and tRNA. A, PSUI forms complexes with both substrate and non-substrate tRNAs. About 10 ng of each 3'-end labeled [^{32}P]tRNA were mixed with PSUI in a total volume of 5.5 ul in TNE buffer. After 3 min at 25^{o}, samples were chilled in ice, mixed with 2.5 ul of glycerol/tracking dye mixture and analyzed by electrophoresis in native 6.5% polyacrylamide gels. Lanes 1-6, S. typhimurium hisT tRNA^{Phe}2, PSUI at 0.0, 0.18, 0.35, 0.53, 0.70 and 1.06 ug, respectively. In lanes 7-18, each tRNA was reacted with 0.0, 0.10 and 0.30 ug of PSUI, respectively. Lanes 7-9, S. typhimurium wild-type tRNA^{His}; lanes 10-12, S. typhimurium hisT tRNA ^{His}; lanes 13-15, S. typhimurium tRNA^{Asp}; lanes 16-18, yeast tRNA^{Phe}. B, Requirements for complex formation. The complete reaction system (8.0 ul total volume) contained 10 ng of [^{32}P]hisT tRNA^{Phe}2 and 0.35 ug of PSUI in TNE buffer with 2 mM dithiothreitol. After incubation at 25^{o} for 5 min, the products were analyzed electrophoretically, as above. Lane 1, tRNA only, no PSUI; lane 2, complete system; lane 3, omit NH_4Cl; lane 4, omit thiol (except for thiol in the enzyme solution); lane 5, omit thiol but add 2.75 mM DTNB.

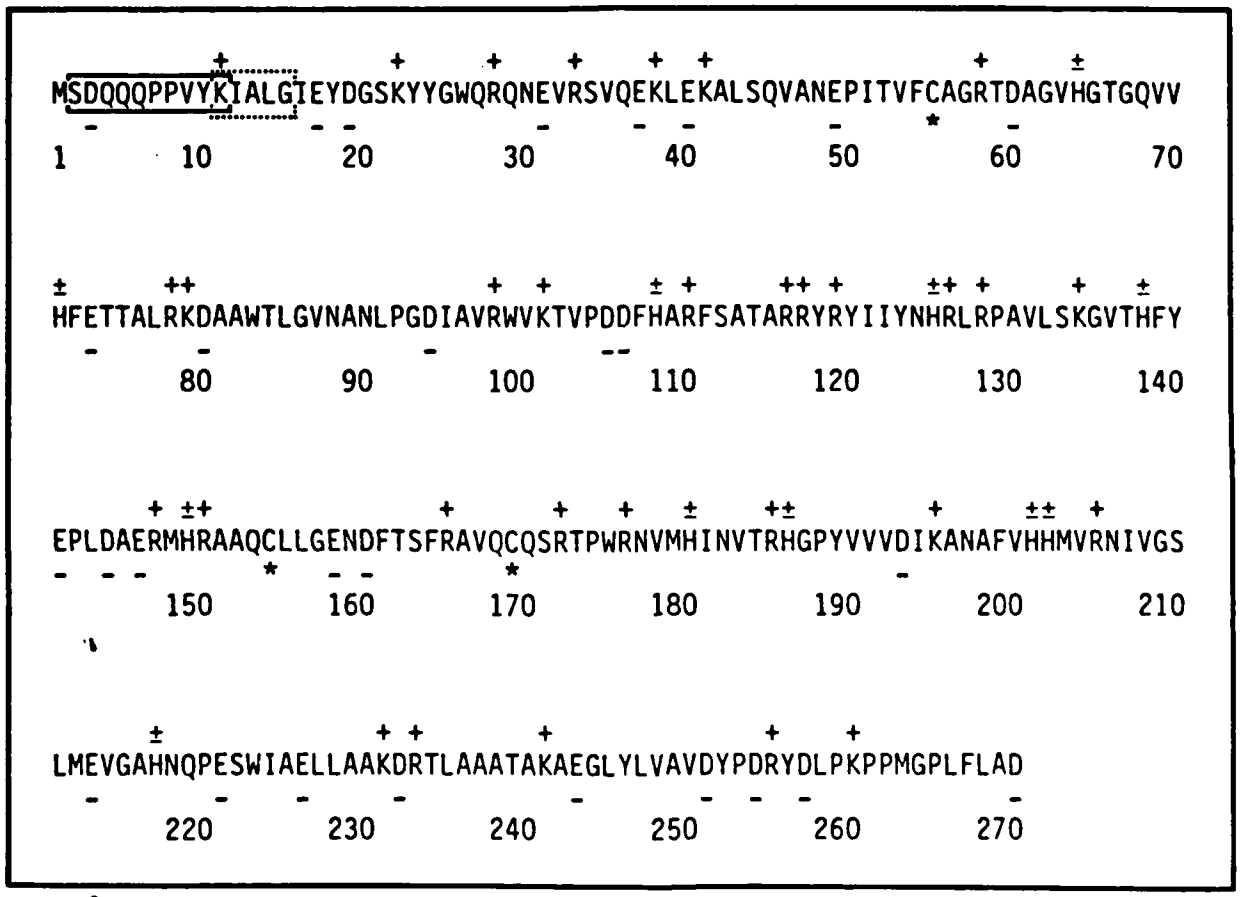
Figure 4. Displacement of bound [^{32}P]hisT tRNA^{Phe}2 by various tRNAs. Complexes were formed by mixing [^{32}P]tRNA (10 ng, 6000 cpm) with 0.5 ug of

PSUI in 5 ul of TNE buffer. After 3 min at 25°, the samples were chilled, 1 ul of water or tRNA chase was added and the samples were incubated for another 5 min at 25° before quenching and electrophoretic analysis. A, Lane 1, [^{32}P]tRNA alone; lane 2, add 1.0 ug of BSA instead of PSUI; lanes 3 and 4, add 0.44 and 0.88 ug PSUI, respectively; lane 5, add 0.88 ug PSUI, displace with 250 ng of E. coli tRNA^{Glu}. B, lane 1, [^{32}P]tRNA alone; lane 2, add 0.88 ug PSUI; lanes 3 and 4, same as lane 2, but displace with 250 ng of wild-type and hisT tRNA^{His}, respectively. C, Displacement of bound [^{32}P]hisT tRNA$^{Phe}_2$ by 10, 25, 100 and 250 ng of tRNA^{Asp} (lanes 3-6) or wild-type tRNA$^{Phe}_2$ (lanes 7-10). The migration of the unbound tRNA is shown in lane 1; that of the bound complexes in lane 2.

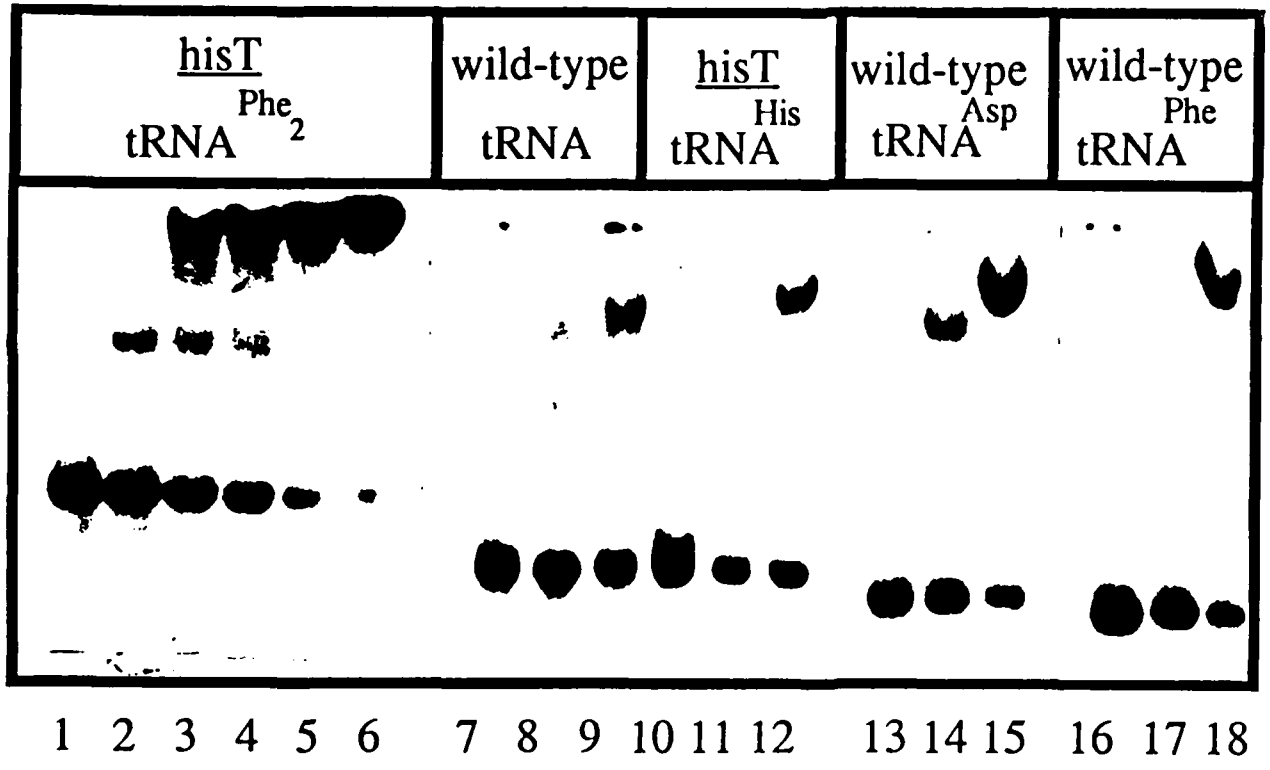
Figure 5. Modification of tRNA^{His}, tRNA^{Tyr} and tRNA^{Leu} isoacceptors by purified PSUI. Bulk, unfractionated tRNA was prepared from E. coli hisT strain FB105. Modification by PSUI was carried out in scaled-up reaction mixtures for incubation periods producing maximum levels of ^{3}H release. PSUI reaction products were recovered by phenol extraction and ethanol precipitation and were stored at -70°. Aliquots of the unmodified and modified RNA were aminoacylated with [^{3}H]tyrosine, -histidine, or -leucine and recovered as described in Materials and Methods. The aminoacylated tRNAs were mixed with wild-type tRNA standards from E. coli 294 charged with the respective [^{14}C] amino acids. The mixtures were analyzed by co-chromatography on RPC-5 columns, using the elution systems given in Materials and Methods. A, modification of hisT tRNA^{His}; B, modification of hisT tRNA^{Tyr} isoacceptors; C, modification of hisT tRNA^{Leu} isoacceptors.

Figure 6. Sites of pseudouridine modification in tRNAs from coliform bacteria. The numbering of residues follows the convention given by Sprinzl

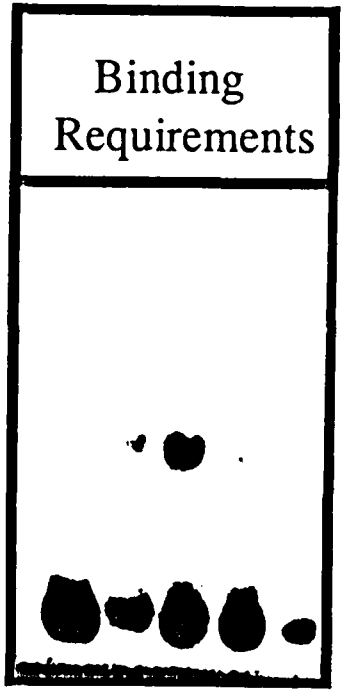
et al. (1). The approximate positions of the glycosyl bonds of modifiable residues are shown by the arrows. (Modified from a drawing by I. Geis).

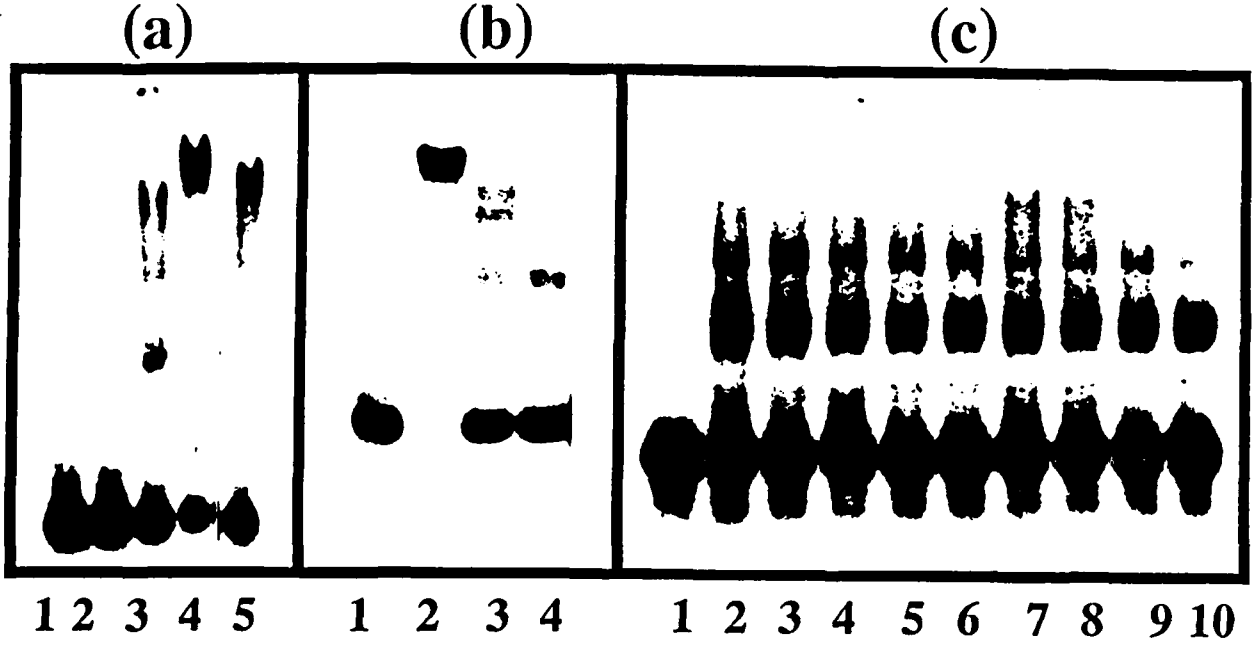

MSDQQQPPVYKIALGIEYDGSKYYGWQRQNEVRSVQEKLEKALSQVANEPITVFCAGRTDAGVHGTGQVV
1 10 20 30 40 50 60 70
HFETTALRKDAAWTLGVNANLPGDIAVRWVKTVPDDFHARFSATARRYRYIIYNHRLRPAVLSKGVTHFY
80 90 100 110 120 130 140
EPLDAERMHRAAQCLLGENDFTSFRAVQCQSRTPWRNVMHINVTRHGPYVVVDIKANAFVHHMVRNIVGS
150 160 170 180 190 200 210
LMEVGAHNQPESWIAELLAAKDRTLAAATAKAEGLYLVAVDYPDRYDLPKPPMGPLFLAD
220 230 240 250 260 270

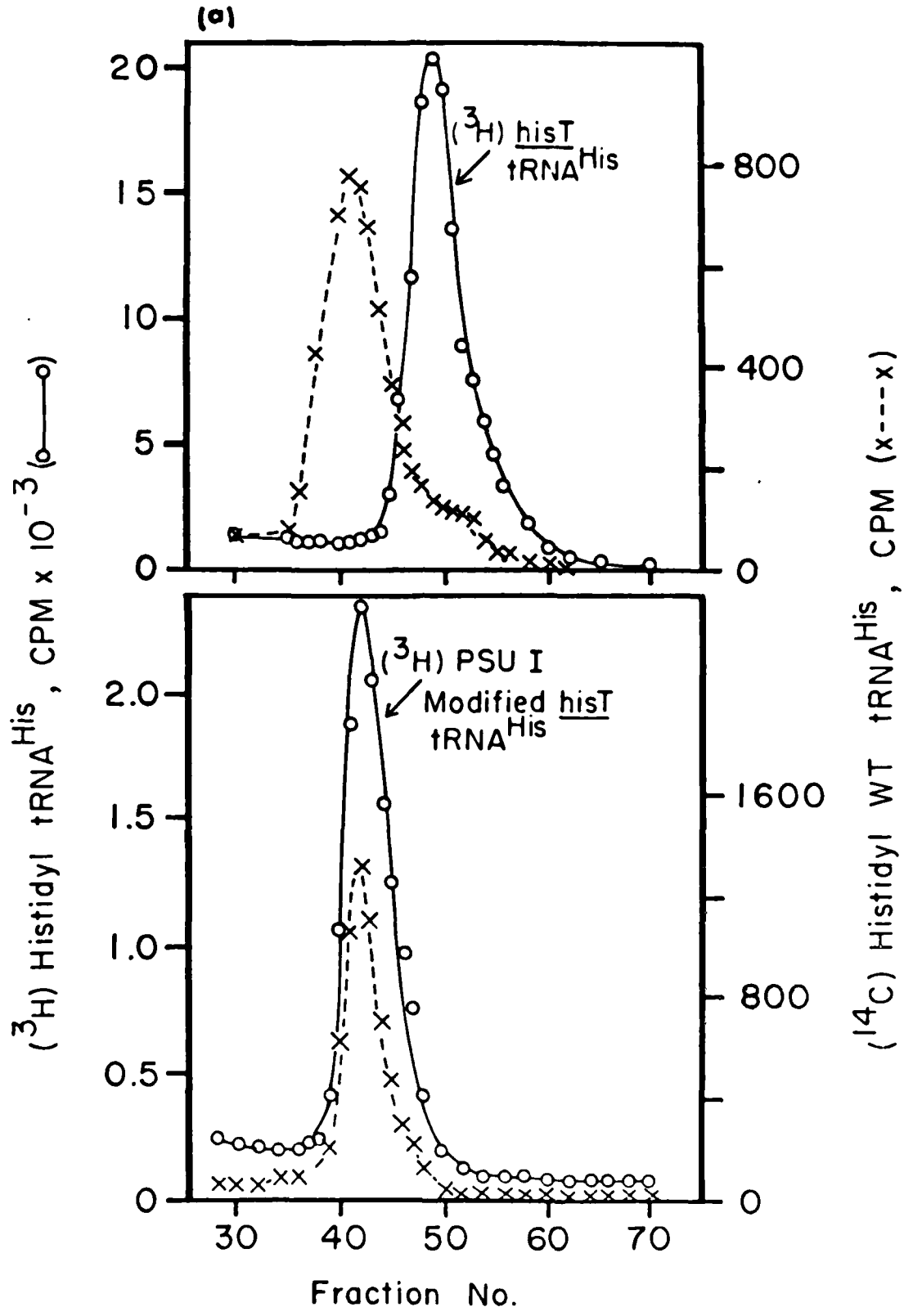
(a)

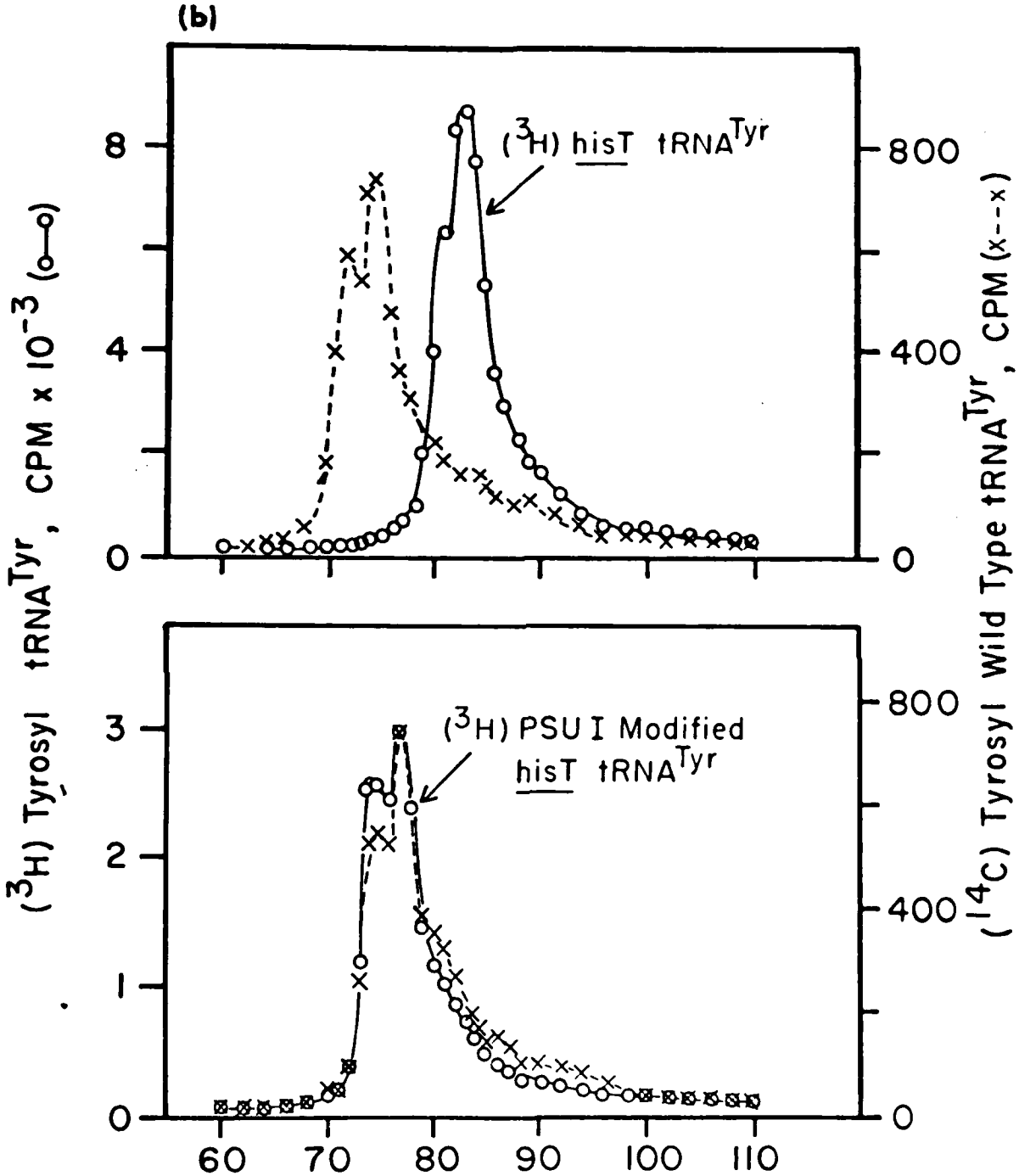

hisT
tRNA Phe 2
wild-type
tRNA
hisT
tRNA His
wild-type
tRNA Asp
wild-type
tRNA Phe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

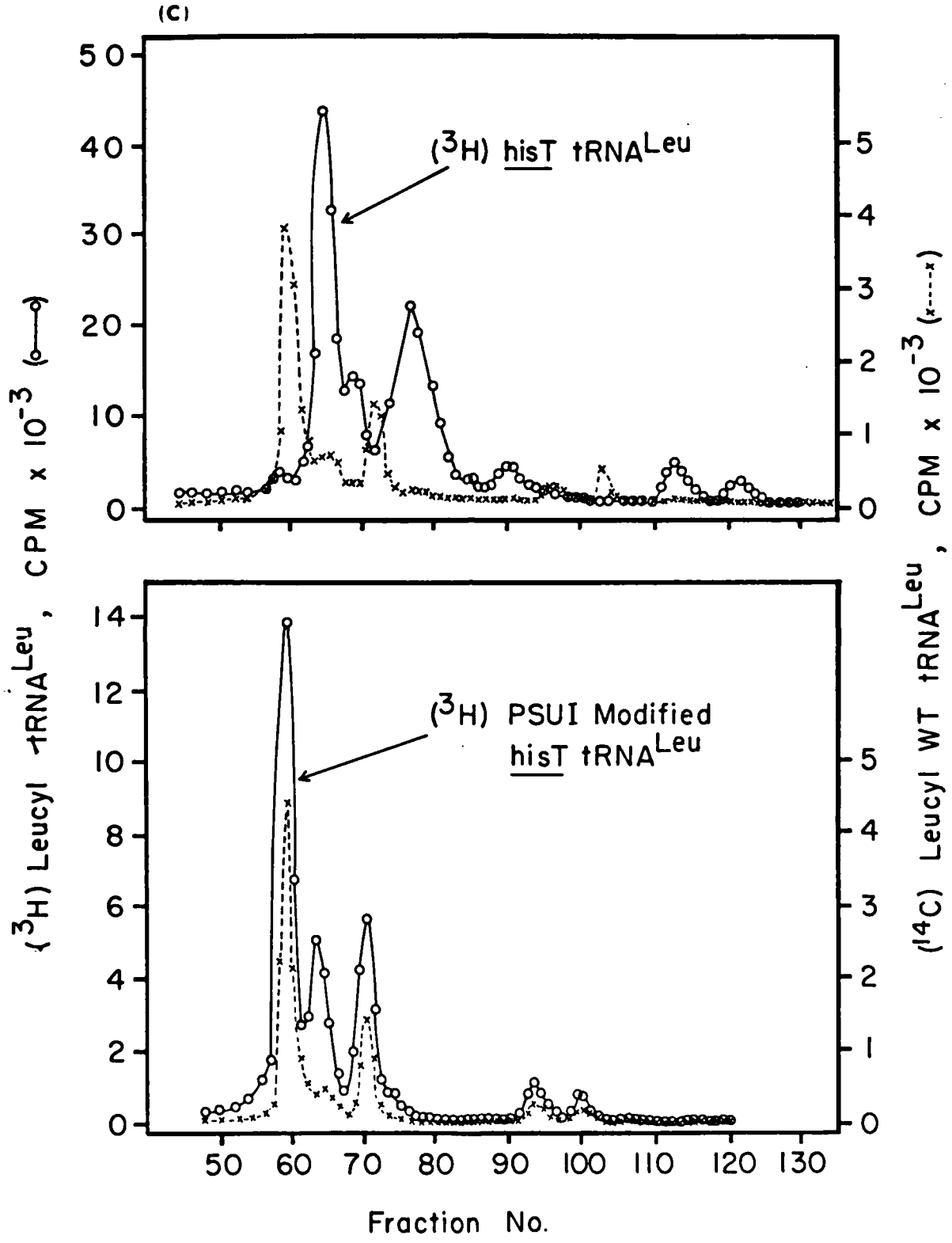
(b)


Binding
Requirements
1 2 3 4 5


(a)
(b)
(c)
1 2 3 4 5
1 2 3 4
1 2 3 4 5 6 7 8 9 10


(a)
20
15
10
5
0
800
400
0
(^{3}H) hisT
tRNA^{His}
2.0
1.5
1.0
0.5
0
1600
800
0
(^{3}H) PSU I
Modified hisT
tRNA^{His}
30
40
50
60
70
Fraction No.
(^{3}H) Histidyl tRNA^{His}, CPM x 10^{-3} (o—o)
(^{14}C) Histidyl WT tRNA^{His}, CPM (x---x)


(b)
(^{3}H) hisT tRNA^{Tyr}
(^{3}H) PSU I Modified hisT tRNA^{Tyr}
(^{3}H) Tyrosyl tRNA^{Tyr}, CPM x 10^{-3} (o—o)
(^{14}C) Tyrosyl Wild Type tRNA^{Tyr}, CPM (x--x)
Fraction No.
0
4
8
400
800
1
2
3
60
70
80
90
100
110


(C)
50
40
30
20
10
0
5
4
3
2
1
0
(^{3}H) hisT tRNA^{Leu}
14
12
10
8
6
4
2
0
5
4
3
2
1
0
(^{3}H) PSUI Modified
hisT tRNA^{Leu}
50 60 70 80 90 100 110 120 130
Fraction No.
(^{3}H) Leucyl tRNA^{Leu}, CPM x 10^{-3} (o—o)
(^{14}C) Leucyl WT tRNA^{Leu}, CPM x 10^{-3} (x----x)

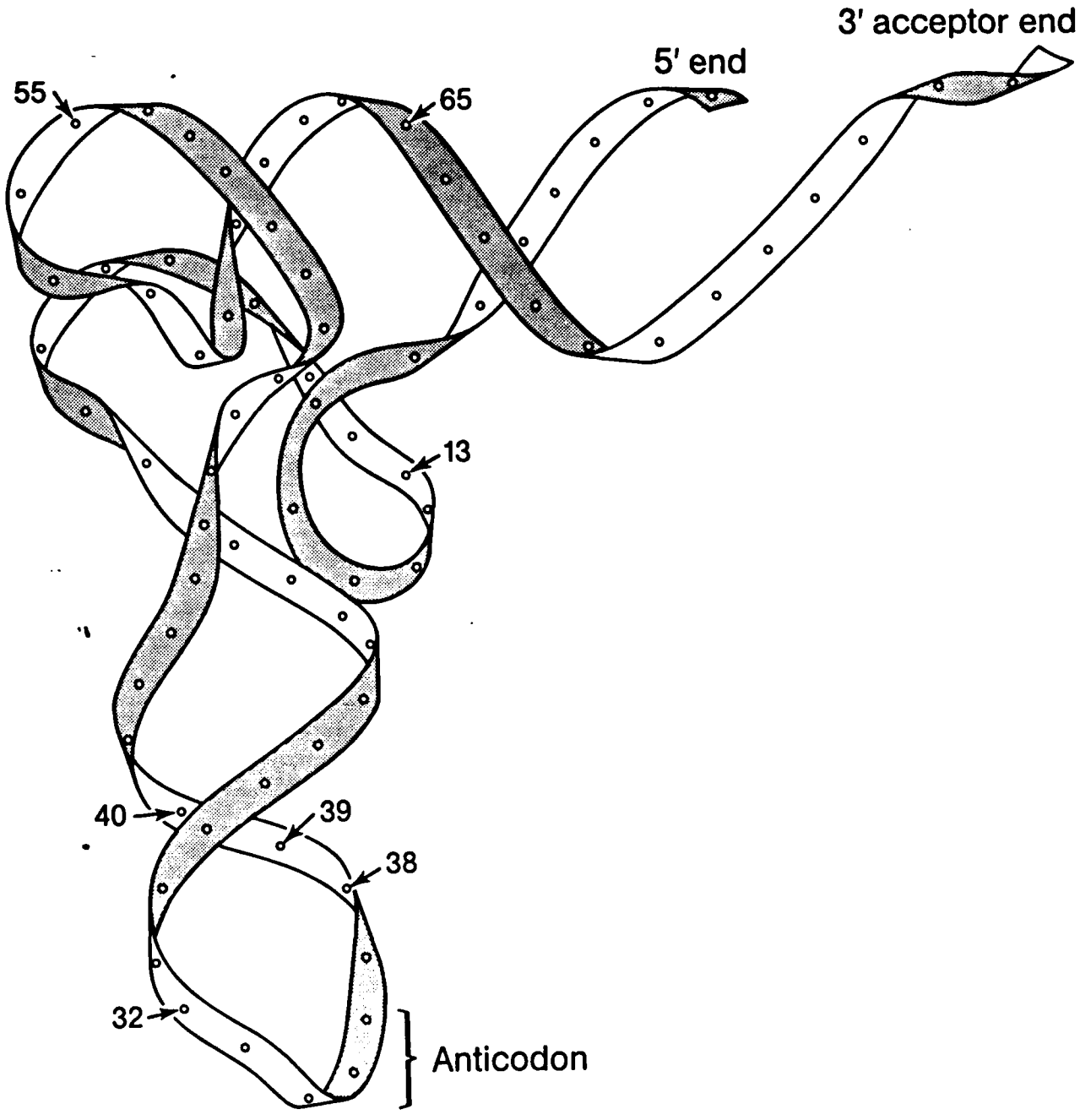

3′ acceptor end
5′ end
55
65
13
40
39
38
32
Anticodon

TABLE II

REQUIREMENTS FOR OPTIMUM RATES OF ^{3}H RELEASE BY PSU I

REACTION SYSTEM	^{3}H Released, CPM	% of Control
Complete System (Control)	1489.	100.
Add pA, pC, pU pG, 0.5 mM each	1342.	90.1
Add ψdo, ψp, 0.5 mM each	1468.	99.1
Add Ura, Udo, pU, UDP, 0.5 mM each	1398.	93.9
Complete System, but [^{3}H] wild-type tRNA in place of hisT tRNA	3.8	0.3
Omit NH_4Cl	10.0	0.67
Omit Dithiothreitol (0.25 uM from enzyme)	1197.	80.4

Incubations were carried out for 20 min at 37° with 8.0 ng of enzyme protein.

TABLE III

THIOL DEPENDENCE OF tRNA ψ SYNTHASE I

REACTION SYSTEM			^3H Released, cpm/20 min assay
Complete System (5 mM dithiothreitol)			1137.
(5 mM Cysteine)			1095.
(5 mM β- MCE)			1136.
Omit thiol (mixture includes 0.25 uM β- MCE from enzyme solution)			764.
Omit thiol, add:			
	DTNB,	0.01 mM	452.
	"	0.10 mM	103.
	"	0.25 mM	24.1
	PCMB,	0.01 mM	445.
	"	0.10 mM	20.8
	"	0.25 mM	0.0
Preincubate Enzyme (minus thiol) with:			
	Buffer only		568.
	DTNB,	0.1 mM	100.
		0.25 mM	19.5
	PCMB,	0.1 mM	100.
		0.25 mM	26.8
	Iodoacetamide,	0.25 mM	86.6

Assay conditions were those described in Table II. For preincubation, 40 ng of PSUI were treated for 15 min at 37° with the indicated additives in enzyme dilution buffer lacking thiol. The samples were chilled and 1/5 of the contents was added to the remaining assay components, including 5.0 mM dithiothreitol. The reactions were continued for 20 min longer at 37°.

TABLE IV

EFFECT OF ADDED NUCLEIC ACIDS ON ^3H RELEASE ACTIVITY

ADDITIONS	A_{260} u.	Expt. # 1		Expt. # 2	
		^3H Release	% of Control	^3H Release	% of Control
Complete System (no additions)		471.	100.	487.	100.
Add *S. typhimurium*					
$tRNA^{His}$ (wild-type),	0.1	65.0	13.8	---	---
$tRNA^{His}$ (*hisT*),	0.1	58.2	12.3	---	---
$tRNA^{Phe}_2$ (wild-type),	0.1	229.	48.6	---	---
$tRNA^{Phe}_2$ (*hisT*),	0.1	67.2	14.3	---	---
$tRNA^{Phe}_1$ (*hisT*),	0.1	75.6	15.5	---	---
$tRNA^{Asp}$,	0.1	383.	81.3	413.	84.8
"	0.2			340.	69.7
"	0.4			251.	51.6
E. coli $tRNA^{Glu}$,	0.1	441.	93.5	453.	93.0
"	0.2			444.	91.0
"	0.4			404.	82.9
$tRNA^{Met}_f$,	0.1	437.	92.7	481.	98.6
"	0.2			398.	81.6
"	0.4			381.	78.2
16S + 23S Ribosomal RNA,	0.1	461.	97.8	---	---
Yeast $tRNA^{Phe}$,	0.1	304.	64.5	340.	70.0
	0.2			252.	51.7
	0.4			165.	33.8
MS-2 RNA,	0.1	479.	102.	495.	102.
	0.2			488.	100.
	0.4			485.	99.4
pBR 322 DNA,	0.1	470.	99.7	---	---

Incubation conditions: 0.1 A_{260} unit of bulk [^3H] *hisT* tRNA; added nucleic acids as shown; 8.9 ng of enzyme protein; incubation for 20 min at 25°.

TABLE V

EFFECT OF tRNA ON ACTIVITY AND STABILITY OF PSU I

REACTION SYSTEM	^3H RELEASED, cpm/20 min	
	Expt. 1.	Expt.2
Complete System (no additives)	1136.	690.
Add FU-tRNA (wild-type), 0.1 A_{260} u.	121.	
Add FU-tRNA (*hisT*), 0.1 A_{260} u.	150.	
Add Yeast $tRNA^{Phe}$ 0.02 A_{260} u.		634.
Add *E. coli* $tRNA^{Glu}$ 0.02 A_{260} u.		678.
Add bulk *Salmonella* tRNA (wild-type) 0.02 A_{260} u.		641.
Add bulk *Salmonella* tRNA (*hisT*) 0.02 A_{260} u.		643.
Preincubate Enzyme (minus thiol) with:		
Buffer only	522.	311.
DTNB, (0.2 mM)		41.4
Dithiothreitol (5 mM)		376.
tRNAs (0.1 A_{260} u each):		
Yeast $tRNA^{Phe}$		622.
E. coli $tRNA^{Glu}$		655.
Bulk *Salmonella* tRNA (wild-type)		649.
Bulk *Salmonella* tRNA (*hisT*)	1143.	653.
FU-tRNA (wild-type)	69.0	
FU-tRNA (*hisT*)	80.8	

The reactions were carried out for 20 min at 25° with 8.0 ng of PSUI protein. Specific activities of the tRNA substrates were 7.0 X 10^6 and 4.1 X 10^6 cpm per A_{260} unit for Experiments 1 and 2, respectively. Preincubations and subsequent reactions were carried out as noted in Table III. The concentration of tRNA during preincubation was 5 times greater than that in the final incubation mixtures.

SUPPLEMENTARY MATERIAL

"PURIFICATION, STRUCTURE AND PROPERTIES OF *ESCHERICHIA COLI* tRNA PSEUDOURIDINE SYNTHASE I"

H.O. Kammen, C.C. Marvel, L. Hardy and E.E. Penhoet

METHODS AND MATERIALS:

Bacterial Growth: *Escherichia coli* strain 294 ($recA^-$), carrying plasmid ψ_{300} was grown in L-broth supplemented with 50 ug/ml ampicillin. *Salmonella typhimurium* strains were maintained on nutrient agar slants and were grown overnight in Vogel-Bonner citrate minimal medium (23) plus 0.02% glucose before transfer to fresh medium containing 0.4% glucose.

Radiolabeled Substrates: Preparation of [5-^{3}H-pyrimidyl] bulk tRNA from the wild-type strain of *S. typhimurium* (LT-2, TA 265) and the *hisT* strain (*hisT* 1504, TA 253) was carried out by growth of the cells to late logarithmic phase in [5-^{3}H]uridine (13). Cell growth, isolation of crude tRNA and its partial purification on DE-52 columns have been described (18).

Radioassay for PSUI Activity: The ^{3}H release assay for PSUI was carried out as described earlier (18), except that reaction mixtures contained 100 mM NH_4Cl and 5 mM dithiothreitol. Incubations were initially conducted at 37° with crude enzyme fractions, but the purified protein frequently showed instability at this temperature. Consequently, for routine assay, reactions were conducted at 25° for 20 min, unless otherwise specified. Enzyme dilutions were made in buffer containing 20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 5.0 mM β-mercaptoethanol, 10% glycerol (w/v) and 0.1% (w/v) bovine serum albumin. The reactions were quenched with 1.0 ml of a slurry of Norit A (12%, w/v, in 0.1 N HCl) and after a minimum of 30 min at 37°, the mixture was filtered through a 0.45 u membrane filter. The non-absorbable ^{3}H in the filtrate was determined by mixing 0.5 ml of filtrate with 4.5 ml of scintillation fluor (Scint A, Packard Chemical Corp.). This sample accounted for an estimated 47.6% of the available Norit filtrate; the total ^{3}H released in individual experiments is corrected for this sampling volume.

Other Enzyme Assays: Assays for tritium release from [5,6-^{3}H]uracil, [5-^{3}H]uridine and [5-^{3}H]UTP were carried out as described for [5-pyrimidyl-^{3}H]tRNA, except that these compounds were present at a concentration of 0.5 mM (25 uCi per ml). tRNA-independent pseudouridylate synthase activity (E.C. 4.2.1.70) was assayed by the method of Solomon and Breitman (24), as well as under conditions optimized for PSUI.

Aminoacylation Enzymes: Aminoacyl synthetase preparations were made from *S. typhimurium* strains LT-2 (*hisT*$^+$) and *hisT* 1504 (*hisT*$^-$) by the method of Kelmers *et al.* (25) and were used to aminoacylate tRNAs from homologous sources. The conditions used for aminoacylation and recovery of aminoacylated tRNA were those of Mullenbach *et al.* (17). Aminoacylated tRNA samples were dissolved in 0.45 M NaCl in MAT buffer (10 mM Na acetate, pH 4.5, 10 mM $MgCl_2$, 2 mM β-mercaptoethanol) and were frozen at -70° prior to chromatographic fractionation on RPC-5.

Analysis of Modification Reaction Products by Reversed Phase Chromatography. RPC-5 resin was prepared by the procedure of Pearson et al. (26) and was packed into a 45 X 1.2 cm column and equilibrated with 0.45 M NaCl in MAT buffer. After sorption of the sample, the column was washed with 40 ml of this buffer and eluted with a 150 ml gradient of NaCl in MAT buffer. The following gradients were employed: tyrosyl tRNAs, 0.45 M to 0.90 M NaCl; histidyl tRNAs, 0.55 M to 1.0 M NaCl; leucyl tRNAs, 0.55 M to 1.2 M NaCl. Chromatography was conducted under pumping pressures that provided a flow rate of ca. 1.0 ml/min. Samples of 1.0 ml volume were collected, mixed with 9.0 ml of Scint A fluor and counted with a Beckman Model 7500 scintillation counter set for dual channel ^{14}C: ^{3}H counting.

Purification of PSUI: E. coli 294 cells containing plasmid ψ_{300} were cultured in L-Broth plus 50 ug/ml of ampicillin and were harvested in late logarithmic phase and frozen at -20°. Pooled cells (150 g) were thawed, suspended in 2.5 volumes of TME buffer (10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 0.1 mM EDTA) and disrupted with a Branson # 185 sonicator (power setting #10, 85 watts) with cooling in an ice-water bath. The procedure was carried out in two 3-min bursts while maintaining the temperature below 10°, by chilling to 3-4° between cycles. The material was centrifuged at 8000 rpm (10,800 X g) in the Sorvall GS-3 rotor for 40 min; the supernatant liquid, including loosely packed material, was recovered and centrifuged for 2.5 hr at 43,000 rpm (200,000 X g) in the Spinco 45 Ti rotor. The hazy yellow supernatants were pooled and frozen at -20° (Fraction 1: Crude Extract).

Fraction 1 was thawed and 1/7th volume of fresh 10% (w/v) streptomycin sulfate, adjusted to pH 7.5-8.0, was added with stirring at 2°. The mixture was centrifuged at 9000 rpm (12,800 X g) and the rubbery pellet suspended in 120 ml of 0.15 M KCl, 0.02 M Tris-HCl, pH 8.0, 0.1 mM EDTA by gentle homogenization. The suspension was stirred for 15 hr at 4° to disperse it evenly (Fraction 2, Streptomycin Pellet).

Cold saturated ammonium sulfate (adjusted to pH 7.5-8 with ammonia) was slowly added to the streptomycin pellet fraction, while stirring at 0-2°; the volume delivered was 2/3 that of Fraction 2. After further equilibration for 10 min, the mixture was centrifuged at 9,000 rpm (12,800 X g) and the supernatant liquid was retained. To this was added another portion of saturated ammonium sulfate (1.2 times the original volume of Fraction 2). After another 10-min equilibration, the viscous mixture was centrifuged in the Spinco 45 Ti rotor for 2 hr at 45,000 rpm. The pelleted material contained about 80% of the activity precipitated by streptomycin sulfate, and was dissolved in TESG buffer (20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 5 mM β-mercaptoethanol, 10% (w/v) glycerol) and dialyzed for 18 hr against two 2-liter changes of this buffer (Fraction 3, 40-65% saturated ammonium sulfate extract).

The dialyzed Fraction 3 was centrifuged to remove insoluble material and was loaded onto a 16.5 X 1.2 cm column of DEAE-cellulose (DE-52), equilibrated with TESG buffer. The column was rinsed with 100 ml of TESG buffer, and was eluted with a salt gradient (400 ml total volume) from 0.0 to 0.2 M KCl in TESG buffer. PSUI activity emerged in a fairly broad peak between 0.05-0.07 M KCl. The pooled fractions were adjusted with solid ammonium sulfate to 80% saturation (56.1 gm per 100 ml of eluate) and the pH brought to 7.0-8.0 with 1 N ammonia. The suspension was centrifuged at 30,000 X g for 30 min and the enzyme pellet was dissolved in 1.0 ml of TESG buffer (Fraction 4, DE-52 eluate).

Fraction 4 was loaded onto a Sephadex G-100 column (85 X 1.2 cm) equilibrated with TESG buffer and was eluted with this buffer at a flow rate of

6.8 ml/hr. Samples of ca. 1.1 ml were collected and assayed for PSUI activity and protein content (Fig 1). The peak tubes were pooled, sorbed to a 0.5-ml DE-52 column equilibrated with TESG buffer, and eluted with small portions of 0.2 M KCl in TESG buffer. Virtually all of the PSUI activity was recovered within the first 1.5 ml (Fraction 5). A portion of the final product was stored at 2^{0}; the rest was adjusted to a concentration of 50% glycerol (v/v) and was stored at -20^{0}.

Preparation of PSUI for Analysis of N-terminal Sequence and Amino Acid Composition: 100 ug (3.3 nmol) of purified PSUI were subjected to electrophoresis in a 12% polyacrylamide-0.1% SDS gel with Tris-glycinate, pH 8.3, containing 0.1% SDS as the running buffer. Electrophoresis was carried out at 140 volts after the tracking dye entered the running gel. A reference lane containing protein standards was excised and stained with Coomassie Blue to localize the the carbonic anhydrase band, whose mobility is virtually identical with that of PSUI (Fig.1). A 5-mm strip of gel containing the PSUI subunit was excised and electroeluted into 2 ml of running buffer for 1.5 hr at 90 volts. The gel eluate was dialyzed against three 3-liter changes of 0.01% SDS over a 20-hour period to remove all traces of buffer and was lyophilized. SDS-polyacrylamide gel electrophoresis indicated a purity greater than 98%. N-terminal sequence analysis of the eluted protein was carried out with a gas-phase sequenator, as described by Hunkapiller et al. (27). For analysis of the amino acid composition, a portion of the sample was subjected to acid hydrolysis and the composition was determined with a Durrum Model D-500 amino acid analyzer (28).

Preparation of Individual tRNA Species: The isolation of $tRNA^{Phe}_{2}$ from wild-type and hisT strains of S. typhimurium has been described by Green et al. (18). $tRNA^{His}$ was prepared from the same sources (wild-type strain LT-2 and hisT 1504) by sequential fractionation with DHB-cellulose, BND-cellulose and RPC-5 chromatography. Yeast $tRNA^{Phe}$, E. coli $tRNA^{Met}f$ and $tRNA^{Glu}_{2}$ were purchased from Boehringer- Mannheim Biochemical Corp.

Fluorouridine-containing bulk tRNA (FU-tRNA) was prepared by growth of S. typhimurium strains in VBC minimal medium containing 0.4% glucose. In early log phase (A_{650} = 0.1), 25 ug/ml of 5-FU and 25 ug/ml of dThd were added. The rate of cell growth declined within 1 hr and the cultures were harvested 4 hr after addition of the analog. Bulk FU-tRNA preparations were isolated in the same manner as preparations lacking 5-FU (18).

3'-End Labeling of tRNA: RNA labeling was carried out by attachment of [5'-^{32}P]pCp with RNA ligase (29). The radiolabeled RNA preparations were purified by electrophoresis in 15% polyacrylamide/ 8 M urea gels and recovered from the gels as described (18). The final RNA preparations were dissolved in sterile water and stored at -20^{0}.

Binding of PSUI to Labeled tRNA: End-labeled tRNA samples, 4000-6000 cpm, were lyophilized and dissolved in 3 ul of TNE buffer (0.02 M Tris-HCl, pH 8.0, 0.1 M NH_4Cl, 2 mM dithiothreitol). Other components and PSUI were added and the mixtures were incubated for 3 min at 25^{0}. The reactions tubes were chilled in ice and 0.5 volume of cold 30% (w/v) glycerol-0.05% (w/v) bromphenol blue was added. Samples were loaded onto a native 6.5% polyacrylamide gel in 0.09 M Tris-glycinate, pH 8.3, in a minigel apparatus (Hoefer Scientific Products). Electrophoresis was conducted for about 40 min at 120 volts, until the tracking dye was ca. 1 cm from the bottom. Gels were stained

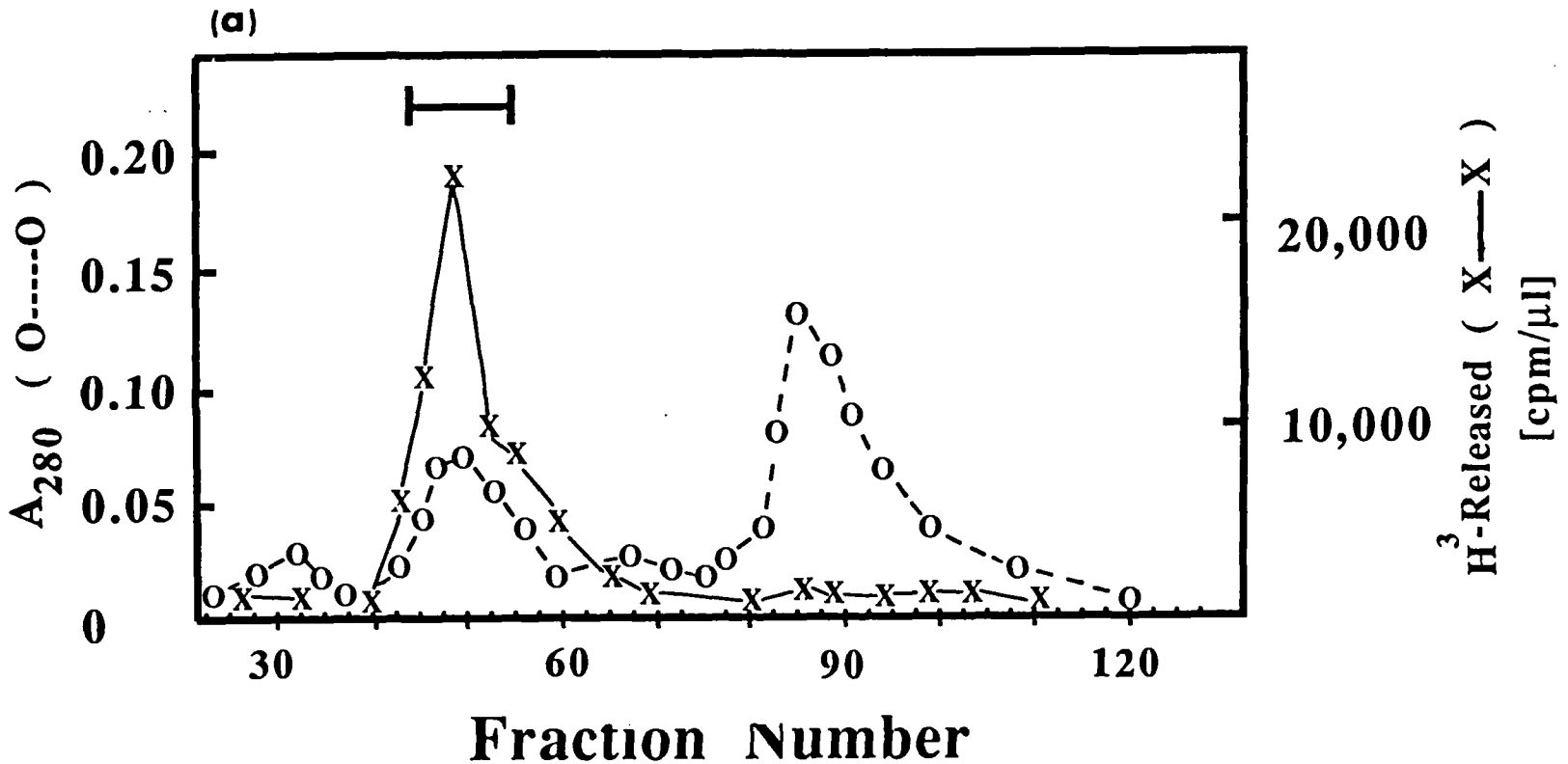
with Coomassie Blue to visualize proteins, then with 0.2% methylene blue in 0.4 M Na acetate (pH 4.5) to visualize RNA. If staining was not required, the gels were dried and autographed at -70° with a DuPont Cronex Intensifying Screen.

Computer Analysis: Analysis of sequence data was carried out using software developed by Dr. Hugo M. Martinez, Dept. of Biochemistry and Biophysics, Univ. of California at San Francisco, San Francisco, CA. Comparisons of the hisT sequence with those in Genbank (release 35.0, 1 August 1985) were conducted with the program "dbalign". Hydrophobicity analysis of PSUI was carried out using the ANALSEQ package of the Whitehead Institute for Biomedical Research, Cambridge, MA. In this analysis the whole protein was surveyed, from amino $acid_1$ to amino $acid_{270}$, using a window size of 21 amino acids.

Materials: Radioisotopes: [5,6-^{3}H]uracil (40 Ci/mmole) was obtained from ICN, [5-^{3}H]uridine (30 Ci/mmol) from New England Nuclear Corp. and [5-^{3}H]UTP (20.9 Ci/mmol), [^{3}H] and [^{14}C] amino acids and [5'-^{32}P]pCp from Amersham-Searle. When used for ^{3}H release assays, samples of the tritiated nucleosides and nucleotides were lyophilized to dryness, redissolved in water and relyophilized again twice to reduce the background of non- enzymatic ^{3}H release from C_5 of the uracil ring. DE-52 was purchased from Reeve-Angel, polyacrylamide gel monomers from Bio-Rad; streptomycin sulfate, ampicillin and 5-halopyrimidines from Sigma. RNA Ligase was obtained from P-L Laboratories and micrococcal nuclease was a gift from Dr. Jeff Hall. All other chemicals were commercial products of the highest available purity.

FIGURE LEGEND

Figure 1. Fractionation of PSUI on Sephadex G-100. See Methods and Materials for details. A, distribution of protein and enzyme activity; B, analysis of column fractions by electrophoresis in 15% polyacrylamide-SDS gels; C, electrophoretic analysis of purified PSUI. Lane 1, buffer only; lane 2, molecular weight standards; lanes 3,4, 1.2 ug of PSUI Fraction 5.


(a)
0.20
0.15
0.10
0.05
0
A_{280} (O----O)
20,000
10,000
^{3}H-Released (X—X) [cpm/µl]
30
60
90
120
Fraction Number

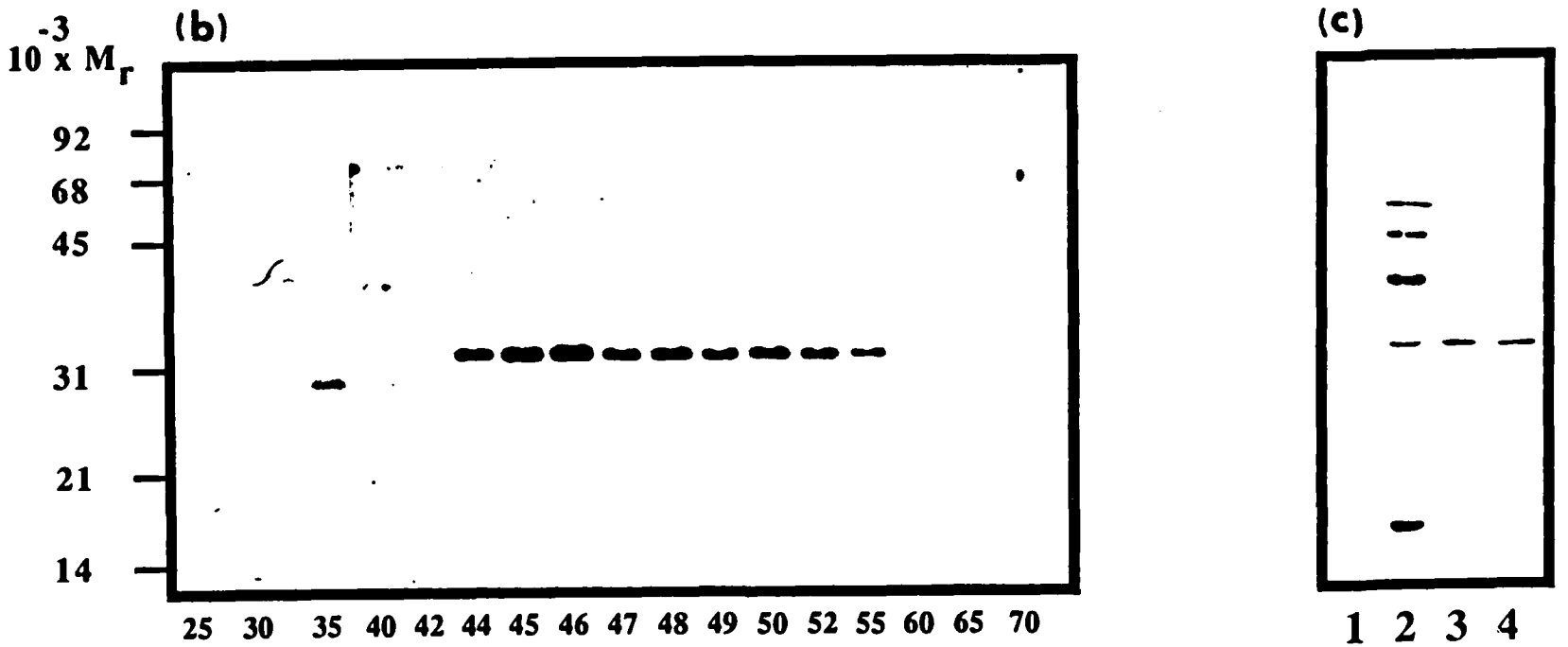

(b)
10^{-3} x M_r
92
68
45
31
21
14
25 30 35 40 42 44 45 46 47 48 49 50 52 55 60 65 70
(c)
1 2 3 4

TABLE I

PURIFICATION OF tRNA PSEUDOURIDINE SYNTHASE I [a]

FRACTION	VOLUME ml	PROTEIN CONC. mg/ml	UNITS [b] ($\times 10^4$)	SPECIFIC ACTIVITY units/mg	FACTOR
1. Crude Extract	400.	22.0	4.14	4.7	1.0
2. Streptomycin Pellet	40.	10.	3.19	79.8	16.8
3. 40-65% saturated Ammonium sulfate	27.	5.5	2.57	172.	36.4
4. DE-52 Eluate	52.2	0.077	0.79	1967.	415.
5. Sephadex G-100 Pool	1.5	0.8	0.42	3465.	730.

[a] The results depicted are from 150 g of pooled frozen cells.

[b] The unit is defined as the enzyme activity that releases 1 nmol of ^3H from hisT [5-^{3}H-pyrimidyl]tRNA under standard assay conditions (20 min incubation at 25°).

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